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The present project is focused on understanding the exact mechanism by which methylation silences gene expression in prostate cancer cell lines. Certain proteins bind preferentially to methylated DNA and these proteins have been shown to repress gene expression. In order to determine which of these protein or proteins interact with methylated genes inside the cells, we plan to use using chromatin immunoprecipitation assay. Two important requirements for this assay include an optimal sonication of the fixed chromatin and quantitative PCR assay for the gene under study. We have determined the linear PCR conditions for the proposed genes as well as the optimal fixation and sonication conditions for the prostate cancer cell lines. Using chromatin immunoprecipitation assay, we have shown that as compared to the AR promoter, the *GSTP1* promoter is enriched in deacetylated histones H3 and H4 in LNCaP prostate cancer cells. We have also found that MeCP2 but not MBD1 or MBD2 interacts with *GSTP1* and *CD44* promoters in LNCaP cells. These results are consistent with the recruitment of histone deacetylase containing complexes by methylated DNA, resulting in a localized deacetylation.

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The progress report covers the period April 1, 2003 - March 31, 2004. However, the distribution of funds was stopped effective October 1, 2002 pending the transfer of Principal Investigator to University of Miami. The transfer of the grant to University of Miami was completed on 2/10/04. Therefore, the report here describes the work performed during the period of 2/10/04 – 3/31/04. We plan to ask for a no-cost extension of a year at the end of this project.

Introduction:

Prostate cancer is the most frequently diagnosed neoplasm and the second leading cause of cancer mortality in men after lung cancer (5). The mechanisms underlying prostate carcinogenesis, tumor progression, and metastatic dissemination remain undetermined. It has been proposed that changes in gene expression through an epigenetic mechanism may be the main force in prostate cancer progression (9). A number of genes have been shown to be aberrantly methylated in prostate cancer cell lines and human prostate cancer tissue specimens (2, 4, 6-8). Direct binding of specific transcriptional repressors to methylated DNA appears to be a major mechanism of transcriptional repression (1, 10). Four methyl-CpG-binding proteins (MBD1, MBD2, MBD3, and MeCP2) have been implicated in transcriptional repression (1) through an interaction with a histone deacetylase complex. Our hypothesis is that methylation represses expression of the proposed genes by the binding of the methylated promoter region by one or more of the methyl-CpG-binding proteins, which interact with a histone deacetylase complex. Numerous studies in animal models and recent studies in humans have demonstrated cancer chemopreventive effects of selenium (3). Our hypothesis is that the mechanism of action of selenium compounds involves demethylation and activation of aberrantly methylated genes in prostate cancer.

Body

Task 1. To determine which methyl-CpG-binding proteins bind to methylated promoters of selected aberrantly methylated genes in prostate cancer cells in vivo (months 1-12):

- Determine linear PCR conditions for the proposed genes (months 1-2)
- Determine optimal fixation and sonication conditions for the prostate cancer cell lines (months 3-6).
- Perform chromatin immunoprecipitation assay (months 7-12).

Chromatin Immunoprecipitation (CHIP) Assay

Formaldehyde cross-linking can be used to rapidly fix in vivo protein-protein and protein-DNA complexes to conserve and analyze native structures. Antibody is used to immunoprecipitate a protein of interest from the whole-cell extract (11, 12). This results in the co-precipitation of any DNA that was directly or indirectly cross-linked to that protein. Reversal of the protein-DNA crosslinks allows isolation and purification of the co-precipitated DNA. A PCR amplification using co-precipitated DNA as template is performed to test whether certain chromosomal sequences have been enriched relative to others. Enrichment indicates DNA sequences that are associated with the protein of interest in vivo.

Two important requirements for this assay are -

1. Optimal sonication of the fixed chromatin - most of the sonicated fragments should be around 500bp.
We have determined
2. Linear PCR conditions for the gene under study

A. Determine linear PCR conditions for the proposed genes (months 1-2)

In the original proposal, we have mentioned that PCR amplification will be carried out with ^{32}P -dCTP in the reaction mixture. PCR products will be separated on a 5% nondenaturing polyacrylamide gel, and bands will be quantitated by phosphorimager analysis. Since a real-time PCR machine at the LSU Health Science Center, Shreveport, LA, became available in the core facility, we have decided to use real time PCR for template quantification. Real-time PCR quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative PCR, which detects the amount of final amplified product

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the endpoint detection by conventional quantitative PCR methods. Real-time PCR quantitation eliminates post-PCR processing of PCR products which reduces the chances of carryover contamination and removes post-PCR processing as a potential source of error. In comparison to conventional PCR, real-time PCR also offers a much wider dynamic range of up to 10^7 -fold (compared to 1000-fold in conventional PCR). This means that a wide range of ratios of target and normalizer can be assayed with equal sensitivity and specificity. It follows that the broader the dynamic range, the more accurate the quantitation.

The real-time PCR system is based on the detection and quantitation of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. There are two general methods for the quantitative detection of the amplicon: (1) fluorescent probes or (2) DNA-binding agents. The TaqMan probes use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in template samples. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe. This ends the activity of the quencher and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye (note that primers are not labeled). The cheaper alternative is the double-stranded DNA binding dye chemistry, which quantitates the amplicon production (including non-specific amplification and primer-dimer complex) by the use of a non-sequence specific fluorescent intercalating agent (SYBR-green I or ethidium bromide). SYBR Green I is a minor groove binding dye. It does not bind to ssDNA.

The threshold cycle or the C_T value is when the system begins to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase. This phase provides the most useful information about the reaction (certainly more important than the end point). The important parameter for quantitation is the C_T . The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the C_T value.

In the last progress report, we had shown the real time PCR standard curve for the Glutathione S-transferase Pi 1 (*GSTP1*) and Androgen receptor (AR) promoters. We have now developed the real time PCR conditions for Endothelin Beta Receptor (EBR) and CD44 promoters.

Primers were designed for the promoter regions of the EBR and CD 44 gene. A standard curve was prepared using serial dilutions of sonicated Human Placental DNA (Sigma) - 160ng, 40ng, 10ng, 2.5ng, 0.625ng, 0.156ng, and 0.039ng. Real time PCR was performed in triplicate on the DNA template. The threshold values (C_T) were plotted against the log ng of input DNA and a regression equation generated. This equation then can be used to quantitate the input quantity of DNA from an unknown sample by using the threshold value. The amplification of the EBR and CD44 promoter was detected Using SYBR Green (BioRad) on a BioRad Icycler

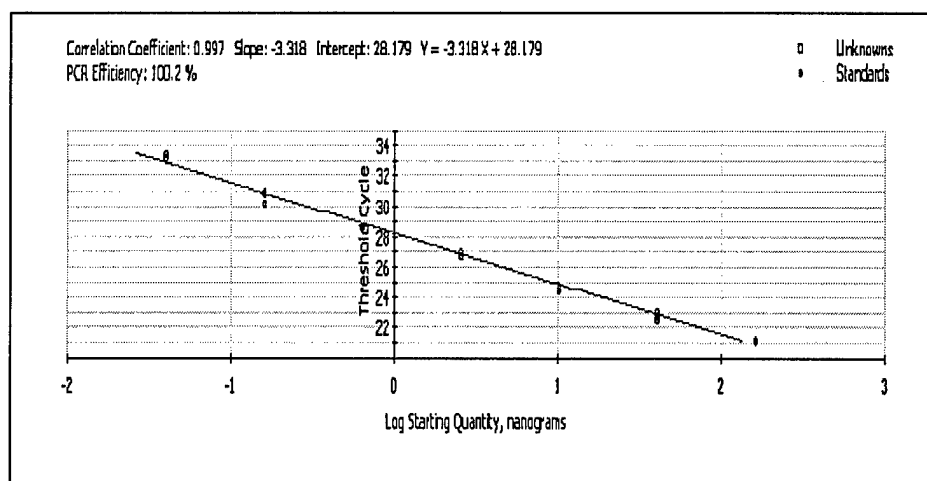


Fig 1 - Standard curve for CD44 promoter

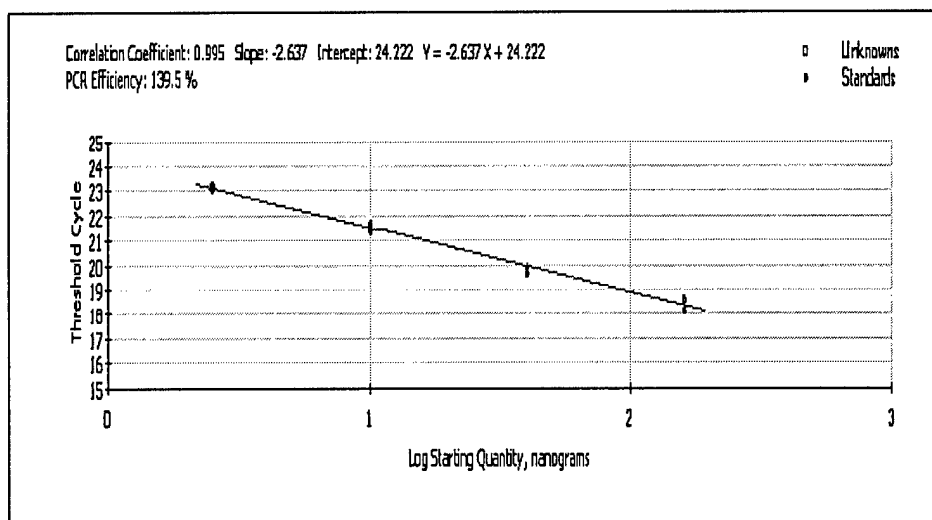


Fig 2 – Standard curve for EBR gene

B. To determine optimal fixation and sonication conditions for the prostate cancer cell lines (months 3-6).

As shown in the progress report submitted last year, we have determined the optimal fixation and sonication conditions for the prostate cancer cell lines.

C. Perform Chromatin Immunoprecipitation (ChIP) Assays (months 7 - 12)

Chromatin immunoprecipitation assays were carried out with a kit from Upstate Biotechnology using the manufacturer's protocol and reagents except that the reactions were scaled down ten-fold. Briefly, 2×10^7 cells were incubated in 0.5% formaldehyde for ten minutes to crosslink bound proteins, washed, lysed in SDS lysis buffer and sonicated to 100-500 bp lengths. Ten μ l chromatin was mixed with 90 μ l dilution buffer and precleared with Protein A agarose, and then the chromatin was incubated with antibody overnight at 4°C. Thirty μ l of Protein A agarose beads was added and the chromatin was immunoprecipitated 2 hours at 4°C. The supernatant (unbound chromatin) and beads (bound chromatin) were separated. The beads were washed five times with the buffers provided and then the chromatin was eluted twice in 1%SDS in 0.1M NaHCO₃. Both bound and unbound chromatin fractions were de-crosslinked by the addition of 5M NaCl and incubation at 65°C for at least 4 hours. Proteins were digested by Proteinase K and then chromatin was extracted with phenol/chloroform. DNA was ethanol precipitated and dissolved in 100 μ l of water. The experiment was repeated on three separate occasions.

Quantitative Real-time PCR - The real time PCR was performed in triplicate using 10 μ l of bound DNA and 5 μ l of unbound DNA template obtained after chromatin immunoprecipitation, the TaqMan or SYBR Green PCR Master Mix (Applied

Biosystems), and primers only (SYBR Green) or primers and probe (TaqMan). When SYBR Green was used, a dissociation curve was created using software from BIoRad to confirm the presence of a single PCR product. Relative quantitation of template DNA was performed as described in User Bulletin #2, ABI Prism 7700 Sequence Detection System (Applied Biosystems).

We have determined the levels of MBD1, MBD2, and MeCP2 at the methylated and silenced *GSTP1* and *CD44* promoters using a chromatin immunoprecipitation assay.

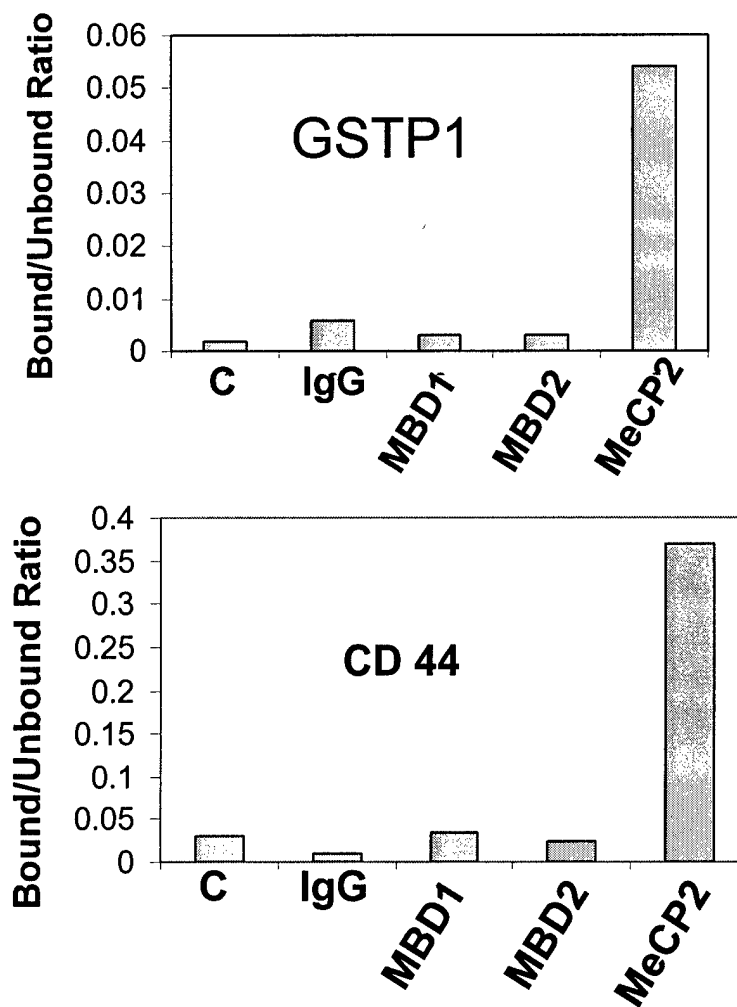


Figure 3. Chromatin Immunoprecipitation (ChIP) Assays. ChIP assays were performed using no antibody (C), non-specific immunoglobulin (IgG), MBD1, MBD2, and MeCP2 antibodies. Enrichment with MeCP2 but not MBD1 or MBD2 antibodies suggests MeCP2 interacts with *GSTP1* and *CD44* promoters in LNCaP cells.

Formaldehyde cross-linked chromatin was immunoprecipitated with antibodies against MBD1, MBD2, MBD3, MBD4 AND MeCP2. The unbound and antibody-bound DNA was analyzed for the *GSTP1* and *CD44* gene promoters using a quantitative real time PCR approach and the ratio of bound to unbound DNA calculated (Figure 3). Enrichment with MeCP2 but not MBD1 or MBD2 antibodies suggests MeCP2 interacts with *GSTP1* and

CD44 promoters in LNCaP cells.

Task 2. To determine if a histone deacetylase complex is involved in transcriptional repression of proposed genes (months 13 - 24):

2a. Effect of TSA treatment on expression of aberrantly methylated genes in prostate cancer cells.

- Cell line growth in varying concentrations of TSA (months 13 -15).
- RTPCR and northern blot for proposed genes (months 13 -16).
- Bisulfite treatment and MS-PCR assay (month 17)

Cell Line growth in Varying concentrations of TSA (months 13 – 15)

Two cell lines, PC3 and DU145, were treated with varying concentrations of TSA (100nM, 300nM, 500nM, 1mM) to see the growth inhibitory effect of TSA. Briefly, the cells were plated at a concentration of 5000 and 10000 cells per well in a 96 well culture plate. Cells were allowed to grow overnight and then varying concentrations of TSA were added and allowed to act for 6 hrs. The TSA containing medium was aspirated out and fresh medium was added to the 96 well plate and cells grown overnight. Cell growth was determined using the MTT based cell proliferation assay kit (Roche). MTT reagent was added to the cells in micro wells and reaction was allowed to proceed for 4 hours at 37°C incubator with 5% CO₂. After 4 hours Solubilization solution was added to dissolve the formazan crystals and the reading was taken at 580nm in an ELISA reader. We found that at concentration of 300 nm and 500 nm TSA was able to bring reduction in cell growth as compared to the control group in PC3 cells. We plan to repeat this experiment to confirm the reproducibility.

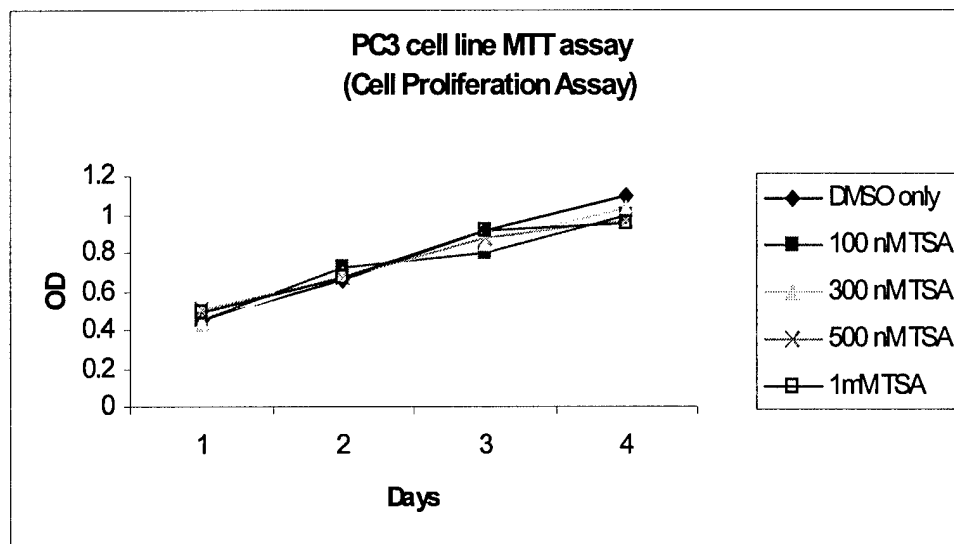


Fig 4 – MTT assay showing effect of varying concentration of TSA on PC3 cell growth

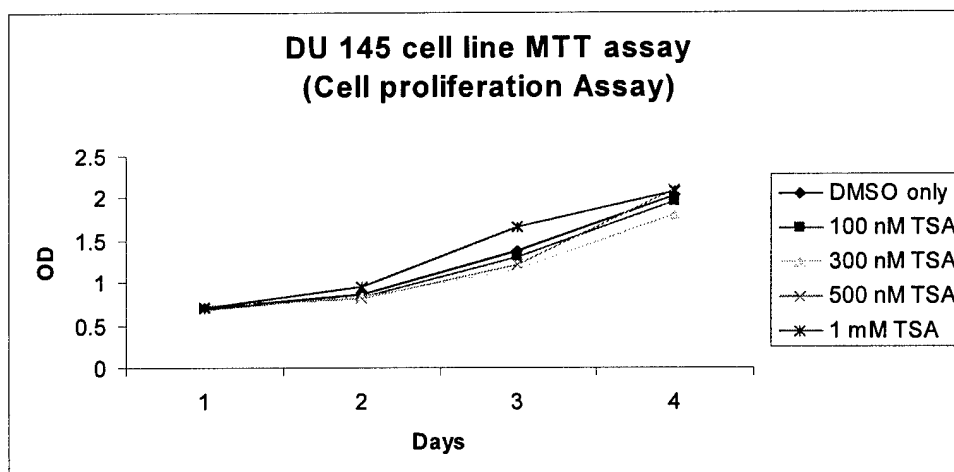


Fig 5 – MTT assay showing effect of varying concentration of TSA on DU 145 cell growth

Key Research Accomplishments

- We have determined the linear PCR conditions for the proposed genes.
- We have performed chromatin immunoprecipitation assay for the GSTP1 and CD44 promoters in LNCaP prostate cancer cell lines for MBD1, MBD2, and MeCP2.
- We have determined the Growth curve on PC3 and DU 145 cell lines in response to the varying concentration of TSA.

Reportable Outcomes

None

Conclusions

We are making good progress on the present project. The progress report covers the period April 1, 2003 - March 31, 2004. However, the distribution of funds was stopped effective October 1, 2002 pending the transfer of Principal Investigator to University of Miami. The transfer of the grant to University of Miami was completed on 2/10/04. Therefore, the report here describes the work performed during the period of 2/10/04 – 3/31/04. We plan to ask for a no-cost extension of a year at the end of this project.

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APPENDICES

None